Molecular analysis of co-infection of Scots pine seedlings with actinobacteria Streptomyces and *Heterobasidion annosum*

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Tiivistelmä – Referat – Abstract

Heterobasidion annosum is a pathogenic fungus that causes extensive damage to many trees in temperate forests including Scots pine (*Pinus Sylvestris*). Various microbes have been studied for potential use as bio-control agents to inhibit or reduce the *H. annosum* infection of trees. This study examined the potential use of bacterial isolates, belonging to *streptomyces* genus, as a bio-control agent for Scots pine seedlings against *H. annosum*. *Streptomyces* species were isolated from surface of mycorrhizal fungi in the forest and they are known to have a mutualistic relationship with mycorrhizal fungi.

The goal of this study was to understand the relationship between *H. annosum* and *Streptomyces* sp. A11. This was tested in in two settings, in presence of pine seedlings, growing in soil and in dual cultures. In the first setting, seedlings from different treatments were inoculated with *Streptomyces* sp. A11, *H. annosum*, co-inoculated with both species or grown in sterile conditions as control. After incubation period, growth and root development of seedlings were analysed. *H. annosum* grown in dual culture against *Streptomyces* sp. A11 was utilised for gene expression using quantitative real time PCR method.

The results indicated that pine seedlings inoculated with both *Streptomyces* sp. A11 and *H. annosum*, had more severe infection compared to the seedlings infected with *H. annosum* alone. This implies that *Streptomyces* sp. A11 can interfere with pine's defence response during interaction with *H. annosum*. Moreover, *Streptomyces* sp. A11 suppressed the growth of *H. annosum* in dual culture. The Suppression of *H. annosum* was potentially because of antifungal secondary metabolites that were produced by *Streptomyces* sp. A11. These secondary metabolites caused disruption in glucose metabolism and cell wall integrity of *H. annosum*. Future experiments should include mycorrhizal fungal species along with species tested in this study. *Streptomyces* sp. are known to respond differently in presence of specific species. The results for this study should be considered for studies of mycorrhizal associated *streptomyces* species and can be built upon for broader future investigations.

Avainsanat – Nyckelord – Keywords

Heterobasidion annosum, *Pinus sylvestris*, *Streptomyces* sp., actinobacteria, mycorrhizal associated bacteria, bot rott disease, actinobacteria, *Streptomyces* sp. secondary metabolites.

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Table of Contents

List of acronyms and abbreviations

BCA	Biological control agents
cDNA	Complementary DNA
dpi	Days post inoculation
ECM	Ectomycorrhizae
EST	Expressed sequence tag
GTPase	Guanosine triphosphatase
HR	Hypersensitive reaction
ISR	Induced systemic resistance
MHB	Mycorrhiza helper bacterium
NFW	Nuclease free water
NO	Nitric oxide
PCD	Programmed cell death
PCR	Polymerase chain reaction
PGM	Phosphoglucomutase
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
Sp.	Species
OTU	Operational taxonomic unit
VOCs	Volatile organic compounds

1. Introduction

Forests have an integral role in the global carbon cycle and consequently climate change. Covering around 31% of the total land area on earth, forests are estimated to absorb about 3 billion tons of carbon per year and store a vast reservoir of carbon. This is double the amount that is in the atmosphere (FRA 2010; Terhonen et al. 2018; Canadell and Raupach 2008). With the potential increase in mean temperature due to climate change, it has been predicted that forests will be subject to higher and more frequent abiotic stress (e.g., extreme weather). Such stresses will favour certain plant pathogens (La Porta et al. 2008; Terhonen et al. 2018). For example, pathogenic fungal species from the genus *Heterobasidion*, which are one of the components of this study, have been observed to increase sporulation and infection frequency in milder winters (La Porta et al. 2008).

With the emergence of phyto-pathogenic fungi and pest insects that are resistant to control agents, (Ntalli and Menkissoglu-Spiroudi 2011) along with the potential increase in forest tree diseases, it is crucial to seek sustainable solutions to counter these issues. Since the green revolution, mineral fertilisers and chemically synthesised pesticides have been extensively used in agriculture and, to a lesser extent, in forestry in order to improve plant's growth and vitality (Rey and Dumas 2017; Trewavas 2001). Decades later, we are experiencing the limits of those strategies for sustainable agriculture and forestry amidst a growing global population: The production of fertilisers requires a large amount of non-renewable energy and the systemic use of chemical pesticides on plants has led to the emergence of resistant pathogens (Rey and Dumas 2017; Bosch et al. 2014).

Emerging technologies are improving our understanding of microbial communities and their complex relationship with other species in ecosystems (Chaparro et al. 2012). Thus, much more is now known about plant-associated microbes and their crucial role in health and development of host plants. Using diverse modes of action and effects, microbes have been observed to benefit plants as bio-fertilizers, phyto-stimulators, plant strengtheners and bio-pesticides (Berg 2009). These beneficial plant-microbe associations provide promising and ecological solution to the limits of using chemicals to maintain healthy plants. The term biological control agent (BCA) is broadly used to define the use of living organisms to control pests, plant pathogens and weeds. Use of

microbes as BCA has been considered an attractive approach without the negative impacts of chemicals (Dukare et al. 2011).

1.1. *Heterobasidion annosum*

Heterobasidion annosum is a fungal species complex belonging to Basidiomycota division. *H. annosum* fungi causes root and stem rot in conifer trees. Although Scots pine (*Pinus sylvestris*) is considered the preferred host, *H. annosum* can infect many other coniferous tree species (e.g. Norway spruce) and few broad-leaved trees as well (Asiegbu et al. 2005a). H. *annosum* is widely distributed in northern hemisphere (Eurasia and North America) temperate forests (Garbelotto and Gonthier 2013). Hence, it is regarded as the most economically damaging pathogen of temperate forests (Asiegbu et al. 2005a).

H. annosum can have a saprotrophic or necrotrophic lifestyle and can switch from one to another depending on the environmental conditions (Garbelotto and Gonthier 2013). In natural forests, *H. annosum* like other saprotrophs, plays a key role in nutrient cycling and wood decomposition. In fact, *H. annosum* is believed to be the most common wood decaying saprotroph of conifers. Moreover, *Heterobasidion* decayed wood provides a niche for various wildlife species and improves the biodiversity of natural forests (Filip and Morrison 1998; Asiegbu et al. 2005a; Garbelotto and Gonthier, 2013). As a white rot fugus, *Heterobasidion* sp. are able to break down all constituents of plant cell wall, including cellulose, hemicellulose, pectin and lignin (Lind et al. 2014).

In order to utilise living wood tissues as source of nutrients, *H. annosum* can switch nutritional mode from saprotrophic to a necrotrophic pathogen lifestyle. Necrotrophs are pathogens that kill living cells in order to feed on dead cells. Biotrophs on the other hand, can only survive by infecting a living plant cells. *H. annosum* can produce both sexual (Basidiospore) and asexual (Conidiospore) spores. Basidiospores have widely been shown to cause infections in nature (Redfern 1998). The infection is mediated by Basidiospores that infect freshly cut stumps or wounds on the stems and roots of a tree (Redfern 1998; Asiegbu et al. 2005a). Germination of adhered spores is facilitated by presence of sugars like glucose, mannose and fructose (Asiegbu 2000). While *H.*

annosum spores seem to not colonise healthy and uninjured trees, the vegetative mycelium of *H. annosum* can infect healthy trees via root contacts or grafts from an infected tree or stump (Asiegbu et al. 2005a). Direct root contact among trees or stumps is essential for secondary infections, as *H. annosum* is not able to grow freely in the forest soil (Garbelotto and Gonthier 2013). Nevertheless, *H. annosum* is remarkably persistent in woody tissues. There are reports of *H. annosum* surviving in a root system of an infected tree for decades and efficiently infecting the next forest generation (Lygis et al. 2004; Stenlid 1987). Moreover, *H. annosum* was shown to inhabit and remain infectious in stumps for up to 62 years (Greig and Pratt 1976; Peri et al. 1990).

H. annosum Spore adhesion can occur within 2 hours after the inoculation of primary roots of juvenile conifer seedlings (Asiegbu 2000). Within the first 24 hours of spore adhesion on non-suberized roots, infection structures such as germ tube and appressoria are developed by *H. annosum* germlings. Penetration of roots occurs by direct penetration through natural openings on root surfaces along with direct enzymatic degradation of root waxes (Asiegbu et al. 2003). Upon colonisation, *H. annosum* secretes a wide range of extracellular enzymes to access and obtain locked up nutrients in host tissues. These enzymes can break down plant constituents such as sugars, pectin, cellulose, lignin and an array of phenolic compounds (Asiegbu et al. 1998; Nord and Hata 1969; Asiegbu et al. 2005a). Various types of root tissues are in contact with pathogen during the colonisation period. These tissues display different characteristics and have different defence properties. Previous studies have demonstrated that root cap and endodermis had the highest resistance to infection whilst, meristem and vascular region were the most susceptible regions of non-suberised roots (Asiegbu et al. 1994; Asiegbu et al. 2005a).

The host plant reacts to *H. anossum* infection by activation of defence signalling pathways with subsequent expression of assortment of defence related genes, which leads to physiological changes along with production of diverse antimicrobial compounds. Large number of enzymes including chitinases, glucanases, and peroxidases are produced to inhibit further growth of the pathogen. These are considered part of pathogenesis-related (PR) proteins (Asiegbu et al. 2005a). At the later stages of infection, phenolic compounds such as phenylpropanoids, flavonoids,

stilbenes and lignans are accumulated to inactivate fungal membranes (Johansson et al. 2004; Nagy et al. 2004; Asiegbu et al. 2005a).

In several biotrophic and necrotrophic pathosystems, the hypersensitive reaction (HR) is triggered in response to pathogen attack. HR leads to induction of defence-related genes, production of phytoalexins and reactive oxygen species and ultimately, programmed cell death (PCD). PCD is carried out by the host plant in cells near infection site as a means to block the pathogen's access to plant cells and nutrients. Unlike biotrophic pathogens, HR barrier does not inhibit the growth of necrotrophic fungi such as *H. annosum* (Mayer et al. 2001; Asiegbu et al. 1998). Therefore, the plant undergoes morphological changes to prevent further penetration and diffusion of fungal toxins and enzymes. Lignification, suberization and papillae formation are examples of morphological changes to create barriers against pathogen penetration (Asiegbu et al. 1998; Solla et al. 2002; Asiegbu et al. 2005a). Peroxidases play a diverse physiological role in host defence: they improve plant defence by mediating the production of toxic radicals and are involved in lignin and suberin synthesis (Asiegbu et al. 1994; Johansson et al. 2004; Nagy et al. 2004).

Despite extensive studies focusing on *H. annosum* in the past 40 years, the development of molecular and genomic studies of *H. annosum*-tree pathosystems have been slow. This can be attributed to *H. annosum* having no known avirulent strain towards conifers and having no genotype from species of Pine family with total resistance against this pathogen (Asiegbu et al. 2005a; Garbelotto and Gonthier 2013). Hence, the primary focus of *H. annosum* disease management has been prophylactic measures to prevent basidiospores to germinate and grow on freshly cut stumps (Asiegbu et al. 2005a). There are number of factors affecting incidences or intensity of the infection such as, site location, soil type and site history (Mattila and Nuutinen 2007; Gonthier et al. 2012; Garbelotto and Gonthier 2013). Higher disease incidences are reported in older stands (Thor et al. 2005). High pH and calcium content have been observed to increase occurrence of disease, as it reduces activity of antagonistic fungi in soil. Furthermore, damage caused by *H. annosum* tends to be higher on fertile soil or sandy soils with low organic matter (Garbelotto and Gonthier 2013). Redfern et al. (2010) study supports this claim by showing that the rate of infection by *H. annosum* is significantly lower in plants growing in peat soils than in those growing on mineral soils.

1.2 Streptomyces

Streptomyces is the largest genus belonging to actinobacteria phylum. Bacteria species from the genus Streptomyces are aerobic, gram-positive with high C-G in their DNA (Loria et al. 2006). Unlike most other bacteria and similarly to fungi, *Streptomyces* species grow as branching hyphae that expands by tip extension. The bacterial hyphae can form a structure called vegetative mycelium. Additionally, *Streptomyces* sp. can form a specialised reproductive structure called aerial hyphae that develops and disperses spores (Flärdh and Buttner 2009). Sporulating lifestyle, increases survivability of *Streptomyces* sp. under harsh conditions and growing as hyphal branch, improves colonisation of plant roots and cells (Seipke et al. 2012). Although *Streptomyces* sp. inhabits a range of niches, they are mostly known to live in soil as successful saprotrophs (Hopwood 2007; Flärdh and Buttner 2009; Loria et al. 2006). Streptomycetes makes up approximately 10% of total soil microbial flora. Hence, they play an essential role in the soil microbial communities (Jannesen 2006).

Streptomycetes are known to produce astonishingly diverse range of natural compounds including many antibiotics. Over 60% of all discovered antibiotics have been isolated from *Streptomyces* species (Liu et al. 2012; Hopwood 2006). As a result, *Streptomyces* sp. have been extensively studied (Chandra and Chater 2014). Over the last decade, the genus Streptomyces in particular, has received tremendous attention from medical and pharmaceutical researchers as a source of numerous bio-active compounds. Apart from antibiotics, other drugs such as antitumor agents, immune-suppressants and antifungals compounds have been isolated from *Streptomyces* species. Consequently, extensive background knowledge of lifecycle and regulation of secondary metabolite synthesis of *Streptomyces* sp. is available (Klementz et al. 2015; Rey and Dumas 2017).

1.2.1 Interaction of *Streptomyces* with other species

By having the ability to produce highly diverse range of bioactive compounds, Streptomycetes can influence microbes and plants that inhabit the same ecosystem. Moreover, *Streptomyces* sp. can also affect the relationship of other species with one

and another (symbiotic, pathogenic, etc.). *Streptomyces* sp. are far more important plant associated bacteria than it was initially recognised (Madhaiyan et al. 2016). *Streptomyces* sp. have a close relationship with plants and fungal species. Evidence suggests that they are able to form close relationship with saprotrophic, pathogenic and mycorrhizal fungi since bacterial filaments can colonise the fungal hyphae and utilise fungal derived substances as source of nutrients (Boer et al. 2005; Poole et al. 2001; Maier et al. 2004; Schrey and Tarkka 2008). Several species have been recognised to influence the symbiosis formed between the host plant and mycorrhizal fungi or host plant and nitrogen fixing bacteria (Palaniyandi et al. 2013).

1.2.2 *Streptomyces* and Plant interactions

Similar to other species, *Streptomyces* sp. can have a wide range of interactions with plants. Many studies have found *Streptomyces* strains that are beneficial towards plants in direct or indirect ways. *Streptomyces* sp. can improve plant vitality and growth by producing an array of phyto-hormones such as auxins, gibberellins, abscisic acid and cytokinins. Production of these phyto-hormones by *Streptomyces* sp. can promote plant growth or alleviate stress. Furthermore, biosynthesis of certain ethylene (plant hormone) inhibiting enzymes by *streptomyces* sp. also reduces stress on host plant (Rashad et al. 2015; Palaniyandi et al. 2014; Rey and Dumas 2017).

Culture filtrate of *Streptomyces olivaceoviridis*, *S. rimosus* and *S. rochei* were shown to enhance crop yield and growth in wheat plants. Further confirming *Streptomyces* sp. capacity in regulating and promoting plant growth using plant hormones (Aldesuquy et al. 1998). Some beneficial *Streptomyces* sp. can mitigate abiotic stress on plants. Sadeghi et al. (2012) reported improved growth and development along with improved nutrient intake of N, P, Fe and Mn in wheat crops inoculated with a *Streptomyces* strain under saline conditions. Co-inoculation of pre-germinated soybean with *S. kanamyceticus*, along with *Bradyrhizobium japonicum*, a native root nodulating bacteria, proved to drastically increase number of formed nodules (up to 55%) and consequently, increase nitrogen composition of plant shoot (Gregor et al. 2003). Despite all the research, little is known about how and where colonisation of the roots by *Streptomyces* sp. occurs (Viaene et al. 2016). Although, the role of host

plants in rhizosphere microbial composition is becoming more apparent. Host plant's selectivity of bacterial communities in rhizosphere, specifically *Streptomyces sp.* was demonstrated by Bakker et al. (2013). The study suggested that the plant's selectivity of bacterial communities in rhizosphere may be mediated through changes in soil environment such as resource availability.

Streptomyces sp. along with other non-pathogenic soil bacteria can activate a form of plant defence named "Induced systemic resistance" (ISR). ISR is mainly not associated with detectable changes in gene expression in plant. However, a more rapid and stronger defence response from plant is observed upon subsequent pathogen encounters (Pieterse et al. 1996; Verhagen et al. 2004; Schrey and Tarkka 2008). For example, Zhao et al. (2012) reported that metabolites produced by *S. bikiniensis* HD-087 were able to effectively supress *Fusarium* Wilt in cucumber leaves and trigger induced resistance in the host.

Apart from forming a relationship with a plant in the soil, certain *Streptomyces* sp. are able to form an endophytic relationship with the host plants (Coombs and Franco 2003). Studying metagenomics of plant microbiota has shown that *Streptomyces* sp. are a major, naturally occurring endophytes that are prone to colonise host plant's roots via the rhizosphere (Rusmana and Lestari 2015; Bulgarelli et al. 2013; Rey and Dumas 2017). Madhaiyan et al. (2016) isolated a novel siderophore producing endophyte (*Streptomyces pini* sp.) from Scots pine needles.

Out of more than 900 described species from Streptomyces genus, only about a dozen of the species are known to be pathogenic towards plants (Bignell et al. 2010). Nevertheless, plant pathogenic *Streptomyces* species have great economic importance for agriculture. For instance, *Streptomyces scabies* and other less virulent *Streptomyces* strains cause potato scab disease worldwide. These scab causing species are neither tissue nor host specific and can infect other tap root crops such as carrot, beet and radish (Loria et al. 2006).

1.2.3 *Streptomyces* and Mycorrhizal fungi

Majority of terrestrial plants develop a beneficial symbiotic relationship with soil-borne fungal species, termed, mycorrhiza. In these close symbioses, host plant provides carbohydrates for mycorrhizal fungi. In return, the fungi improves accessibility of the plant to water and nutrients in the soil via its complex hyphal structures (Hampp and Schaeffer 1999; Smith and Read 2010; Maier et al. 2004) Most trees from temperate forests develop a form of mutualistic relationship called Ectomycorrhizal (ECM) symbiosis (Kurth et al. 2015). ECM fungi play a crucial role in nutrient cycles, growth and stress mediation of forest trees and woody shrubs. In addition to ECM improving hosts' access to nutrients, fungal mycelium provides pathway for translocation of organic carbon to micro-environments in the soil (Marupakula et al. 2016). Mycorrhizospheres also contain diverse bacterial communities (Schrey et al. 2012). Bacteria are ubiquitous in ectomycorrhizosphere and they can colonise on inter or intracellular locations within the mantle and Hartig net of the fungi. Considerable bacterial biofilms has also been observed on the foraging hyphal front (Nurmiaho-Lassila et al. 1997; Poole et al. 2001). The term 'mycorrhiza helper bacterium' (MHB) has been used for any species of bacteria that is able to promote mycorrhizal symbiosis formation. Number of isolated *Streptomyces* sp. are considered MHB (Frey-Klett et al. 2007). A Study showed that two MHB Streptomycetes were able to significantly improve mycelial growth and mycorrhizal formation rate in both Spruce and Pine with their appropriate mycorrhizal fungi (Schrey et al. 2005; Schrey and Tarkka 2008).

Poole et al. (2001) demonstrated that bacteria species isolated from ectomycorrhizosphere were able to stimulate mycorrhizal formation between fungi *Lactarius rufus* and *P.sylvestris*. This can be done by variety of mechanisms such as, increase host's root receptivity (induced by bacteria) to fungal infection or by direct growth stimulation of fungi (Poole et al. 2001). Despite a number of studies demonstrating the importance of MHB for establishing the mycorrhizal symbiosis, the role of plant in response to MHB, in presence or absence of the ECM, is not well known (Kurth et al. 2015). It has been proposed that changes in plant growth rates when co-inoculated with ECM and MHB may be due to associated modification of main pathways such as Ethylene signalling and plant hormones such as auxin (Vacheron et al. 2013; Kurth et al. 2015). Overall, it can be stated that some

Streptomyces sp. and mycorrhizal fungi have an intricate and selective relationship. Various studies have demonstrated that *Streptomyces* sp. can either promote or supress mycorrhizal formation in various plants (Ames et al. 1984; Ames 1989; Schrey and Tarkka 2008)

1.2.4 *Streptomyces* and plant-pathogenic fungi

Streptomyces sp. have a big role in suppressing other micro-organisms in the soil due to their ability to produce diverse range of secondary metabolites. This includes suppressing phyto-pathogenic microbes, particularly, fungal species. These antagonistic properties are indirectly beneficial towards plants as it reduces the risk of pathogen infection. *Streptomyces* sp. are known for their antifungal properties (Weller et al. 2002). It has been reported that about one third of *Streptomyces* sp. from humus layer of Norway spruce plantation contain antifungal properties while none of the strains promoted the growth of pathogenic fungi (Elo et al. 2000; Schrey et al. 2012). *Streptomyces* sp. also display specific patterns of inhibition towards other *Streptomyces* strains and other bacterial species (Schrey et al. 2012). Earlier studies recognised certain species of *Streptomyces* for their ability to produce cell wall degrading enzymes such as Chitinases, cellulases, hemicellulases and glucanases (Yuan and Crawford 1995; Trejo-Estrada et al. 1998). Shekhar et al. (2006) discovered that presence of chitin in growth media of *S. violaceusniger* XL-2 has a role in activating production of endochitinase, a bio-control protein that can breakdown fungal cell wall. Volatile organic compounds (VOCs) are also part of diverse secondary metabolites that are produced by *Streptomyces* sp. Isolated VOCs from several Streptomycetes have been shown to inhibit hyphal growth of pathogenic fungi and enhance plant root-shoot biomass (Cordovez et al. 2015).

Another antagonistic mechanism of microbes in rhizosphere is competition for iron, as iron is essential for growth. Yet, iron concentration in rhizosphere is extremely low (Pal and Gardener 2006). Microbes produce iron chelators called siderophores. Their role is to mediate iron uptake by the microbial cell whilst reducing iron availability for other microbial species (Palaniyandi et al. 2013; Winkelmann 2002). Antagonism of three separate *Streptomyces* species against pathogenic fungi *Moniliophthora*

perniciosa was observed by Macagnan et al. (2008). Inhibition of basidiospores germination were more evident in iron deficient medium, suggesting that siderophores play a role in antagonisms between the *Streptomyces* sp. and some phytopathogens.

Suppression of pathogens by *Streptomyces* sp. is not limited to root infections. Studies have demonstrated the application of antibiotic producing Streptomycetes as bio-control for foliar diseases (Palaniyandi et al. 2013). The *Streptomyces* sp. were used to supress diseases such as alfafa spot caused by fungal pathogen *Phoma medicaginis var. medicaginis* or anthracnose (*Colletotrichum gloeosporioides*) in yam (Palaniyandi et al. 2011; Samaca et al. 2003). From all the mentioned studies, it appears that *Streptomyces* sp. have high potential to be used as bio-control agents.

1.3 Gene expression and Transcriptomics

Transcriptional profiling is described as simultaneous analysis of expression of large number of genes in a species, providing an overall picture of gene expression at the time of sampling. Using this method to study tree-microbe interactions is a valuable source of information for better understanding responses of species on gene level (Kovalchuk et al. 2013). Moreover, utilising the rapidly advancing genomics technologies, leads to selection for microbial agents with more predictable and consistent effects (Berg 2009).

Quantitative real time PCR (RT-PCR) is now considered a standard method to quantify the nucleic acid level from large variety of samples. Currently, RT-PCR technique is more widely used compared to other methods (e.g., northern blot) due to it being relatively faster, more sensitive and less laborious to perform (Raffaello and Asiegbu 2013).

Relatively small amount of genetic information is needed for application of RT-PCR. Consequently, RT-PCR is considered a powerful tool particularly in research fields such as forestry where genomic information for many tree species are still quite limited (Groover et al. 2007; Raffaello and Asiegbu 2013). For designing primers, having either a draft genome assembly, an expressed sequence tag (EST) library or a nucleotide sequence coming from closely related species, could provide enough

information to run a qPCR experiment (Raffaello and Asiegbu 2013). Nonetheless, precise protocols must be followed for every step of the qPCR process including data acquisition, and analysis (Bustin et al. 2009). Additionally, qPCR method requires to include validated reference genes of the species that is being studied to be used in a data normalisation step (Raffaello and Asiegbu 2013).

Transcriptional profiling of tree responses to pathogen attack of variety of tree-pathogen systems have been studied including Scots Pine and *H. annosum* (Adomas et al. 2007; Asiegbu et al. 2005b; Kovalchuk et al. 2013). Transcriptome profiles of *H. annosum* has also been analysed in previous studies (Raffaello et al. 2014; Chen et al. 2015). Lehr et al. (2009) similarly investigated difference in gene expression in *H. annosum* and *H. abietinum* treated with antifungal substances produced by *Streptomyces* sp. Ach 505.

In this study, the initial aim was to perform qPCR tests on both pine seedlings samples from the microcosm setup and on *H. annosum* fungi harvested from antagonistic dual culture with *Streptomyces* sp. A11. Numerous transcriptomic studies of Scots pine, *H. annosum* and pine - *H. annosum* pathosystems provides fundamental information required for efficient experimental design in this study and they provide valuable sources for result comparisons.

1.4 Antagonistic interactions

Over the recent years, a number of studies have particularly focused on interaction of *Streptomyces sp.* isolated from rhizosphere (or ectomycorrhizosphere) and pathogenic species mainly from *Heterobasidion* genus and in some cases, in presence of host tree (Mainly conifers).

Maier et al. (2004) isolated gram-positive bacteria from rhizosphere of Norway spruce (*Picea abies*) stand rich with ECM fungi, *Amanita muscaria* fruiting bodies. Based on *in vitro* studies of antagonism, it was shown that the isolated actinomycete significantly promoted the growth of ECM fungi *A. muscaria.* Additionally, the isolated actinobacteria was able to inhibit the growth of spruce pathogenic fungal

species such as *Armillaria obscura* and *H. annosum*. This particular isolate was found to belong to *Streptomyces* genus and was named *Streptomyces* AcH 505 (Maier et al. 2004). Hence, this novel strain is considered a MHB and it has been focus of subsequent studied. Schrey et al. (2005) further confirmed growth promotion of forest ECM fungi by *Streptomyces* AcH 505 and another *Streptomyces* strain. Both of the *Streptomyces* species demonstrated the ability to promote mycorrhizal formation and fungal growth by inducing the expression of several genes in ECM fungi.

Riedlinger et al. (2006) discovered a novel fungal growth promoting compound named auxofuran from *Streptomyces* AcH 505. Evidently, auxofuran stimulates growth of both mycorrhizal and pathogenic fungi. Moreover, two antifungal substances named WS-5995 B and WS-5995 C were also identified from the same species. Further studies showed that sensitivity or resistance of different fungal species (pathogenic and mycorrhizal) towards the antifungal substances is directly influenced by fungal growth or suppression in presence of *Streptomyces* AcH 505.

Lehr et al. (2007) continued this line of study by testing 12 different isolates of *Heterobasidion* sp. and their ability to colonise Spruce seedlings in presence of the *Streptomyces* AcH 505. The bacterium was able to suppress the root colonisation of spruce by 11 out of 12 tested *Heterobasidion* isolates. The 12th isolate, *Heterobasidion abietinum* 331 was not only not suppressed by but surprisingly, *Streptomycete* AcH 505 promoted root colonisation of the seedling by the fungal pathogen. Further examination showed that this was done by suppression of peroxidase activity in spruce seedlings rather than growth promotion of the pathogen. *H. abietinum* 331 also showed tolerance to antifungal substance (WS-5995 B) produced by AcH 505 (Lehr et al. 2007). This demonstrates the complex and specific relationship of *Streptomyces* sp. and other species in different conditions. To examine these relationships more extensively, Lehr et al. (2008) performed a similar study with another *Streptomyces* strain (*Streptomyces* sp. GB 4-2) against *Heterobasidion abietinum* and spruce seedlings. Results showed that the *Streptomyces* strain simultaneously promoted spore germination and hyphal growth of pathogenic fungi as well as, induction of both local and systemic defence response in Spruce (Lehr et al. 2008).

Studying wider range of Streptomyces strains and fungal (pathogenic and mycorrhizal) species, Schrey et al. (2012) concluded that different strains of mycorrhiza associated

Streptomycetes, isolated from same mycorrihzosphere of Norway spruce, have specific inhibitory characteristics against different fungal species. This is due to structurally diverse secondary metabolites produced by each *Streptomyces* species. The examined secondary metabolites of each *Streptomyces* strain in the study, contained variety of antifungal and antibacterial compounds along with siderophores.

Golinska and Dahm (2013) performed a number of *in vitro* and *in vivo* experiments studying antagonistic properties of isolated *Streptomyces* sp. from bulk soil and rhizosphere against fungal phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani* in relation to pine seedlings. Both of the fungal pathogens can cause pine seedling damping-off. In most of the experiments, the inhibitory effects of Streptomycetes on fungi (in varying degrees) were observed. Although, in some cases, fungal growth was stimulated by certain bacterial strains (Golinska and Dahm 2013). Pine seedlings in the *In vivo* part of the experiment were set up in both sterile and non-sterile soils. The result showed that the positive effects of *Streptomyces* sp. were more evident in non-sterile soil (Golinska and Dahm 2013). Inoculation of pine seedlings with actinomycetes alone (isolated from pine forest) had no negative impact on seedling growth yet, the positive effect on seedlings were small (Golinska and Dahm 2013).

Based on all the mentioned studies, it seems that *Streptomyces* species have a very distinct role in the rhizosphere. *Streptomyces* sp. have the capacity to produces an array of metabolites that can concurrently stimulate and suppress fungal growth, as well as improve or weaken plants defence response towards pathogens. Additionally, these responses are highly strain specific hence, we cannot predict interactions of other strains of *Streptomyces* with *Heretobasidion* species based on previous similar studies.

2. Objective of the study

2.1 Central questions

The main objective of the study is to have better understanding of the relationship between pathogenic fungi *H. annosum* and the mycorrhiza-associated bacteria, *Streptomyces* sp. A11. The interaction of these two species was studied in two settings, in presence of pine seedlings growing in soil and in dual cultures. This study primarily investigates the following:

1. The effects of *Streptomyces* on growth of Scots pine seedlings, solely and in co-inoculation with *Heterobasidion.*

2. The effects of *Streptomyces* on root development of Scots pine seedlings, solely and in co-inoculation with *Heterobasidion.*

3. Differentially expressed genes of *H. annosum* in response to *Streptomyces* metabolites secreted in dual culture interaction.

2.2 Hypotheses

Hypothesis 1: Inoculation of Pine seedlings with Streptomyces sp. A11 will increase overall growth and root development of the plant.

This was tested in soil microcosm experiment by comparing the results of samples from *Streptomyces* sp. A11 inoculation with control treatments.

Hypothesis 2: Inoculation of Pine seedlings with Streptomyces sp. A11 protects it from deleterious effects by H. annosum root infection than seedlings without Streptomyces root inoculation.

This was tested in soil microcosm experiments by comparing the results of samples from *H. annosum* inoculation with samples from co-inoculation treatment.

Hypothesis 3: Interaction of H. annosum in dual culture plate against Streptomyces sp. A11 leads to differential gene expression of the pathogen

This was tested using collected data from gene expression of *H. annosum* from the dual culture experiment setup.

3. Material and methods

3.1 Experimental materials

Three *Streptomyces* strains were previously isolated and kindly provided by Dr. Lu-Min Vaario. *Streptomyces* sp. B2 (LC420152) and *Streptomyces* sp. FY4 (LC475423) were separately isolated from *Tricholoma matsutake* mycorrhizal fungi directly from shiro soil (Wakayama Prefecture), and a matsutake mycorrhizas from a 6-month container seedling of *Pinus densiflora* on the forest soil, Tokyo (Vaario et al. 2018). *Streptomyces* sp. A11 was isolated from *Suillus luteus* mycorrhizas from a 6-month container seedling of *P. densiflora* on the forest soil, Tokyo (Vaario et al. 2019). All *Streptomyces* strains were grown on ISP2 agar (Shirling and Gottlieb 1966) and incubated at 28°C.

Heterobasidion annosum (isolate 02034) was kindly provided by the forest pathology lab in University of Helsinki. It was grown on 2% Malt extract plates and incubated at 25°C.

Pinus sylvestris (Scots Pine) seeds were provided by the forest pathology lab (Seed code: E2128). They were stored at -20°C Freezer until use.

3.2 Screening of *Streptomyces* sp. for their ability to inhibit the growth of *H. annosum*

In order to select a *Streptomyces* sp. with highest antagonism, a preliminary dual culture experiment was set up. Considering *Heterobasidion* fungi and *Streptomyecs* sp. have different preferred growth substrates, the dual culture was performed on an array of mediums.

Each plate was set up with same size plug from fungi (from edge of growing hyphae) and bacteria culture with set distance from one and another and the edge of plate. Dual cultures were set up on Malt extract (2%), MMN (Marx 1969), ISP2 and Hagem (Stenlid 1985) media with six replicates for each sample. Plates were incubated in the dark at 25°C for 14 days. After incubation, radial growth of *Streptomyces* agar plug to

the edge of grown fungal hyphae was measured. Differences in the length of inhibition zone from each plate were statistically analysed.

The series of experiments can primarily be divided into two setups:

1) Soil microcosm setup with Pine seedlings, *H. annosum* and *Streptomyces* sp. A11 (Chapter 3.3).

2) Agar plate dual culture setup with *H. annosum* and *Streptomyces* sp. A11 (Chapter 3.4).

3.3 Soil microcosm setup

3.3.1 Seed Preparation

Pine seeds were cleaned with washing liquid soap and placed under running water for 20 minutes. They were then placed in 70% ethanol for 30 seconds followed by being submerged in 30% hydrogen peroxide for 5 minutes. Seeds were then air dried under laminar flow. Surface sterilised seeds were then sown to a 1.5% water agar plate with no carbon source and incubated in the dark. Once seeds began to germinate, the plates were incubated in a growth chamber with a photoperiod of 16 h at 20℃ for 14 days prior to planting in soil.

3.3.2 Soil Preparation

Soil used in this study was extracted from a pine-spruce mixed forest at Nuuksio national park. The soil was sieved through a 2mm sieve and mixed with vermiculite in 1:1 ratio. The soil mixture was dried overnight at 105℃. An appropriate amount of distilled water was added to ensure uniform 20% moisture in throughout the soil containers. The soil mixture was sterilised by autoclaving the soil three times, with one-day intervals between each autoclave.

3.3.3 Microcosm set up

In order to grow seedlings in a sterile environment (prior to inoculations), rectangle petri dishes (EIKEN CHEMICAL Co. Ltd, Japan) with measurements of 230×80×14.5mm were used as the microcosm. Two or three holes were made on the plates to allow air flow. The holes were sealed using MilliSeal™ hydrophobic fluoropore PTFE Membrane (Figure 1).

The same amount of sterile soil was added into each plate. Three newly germinated seedlings (two weeks after pre-sown on agar) were then planted in each plate. These plates were then sealed using Parafilm®. In order to stimulate darkness, the exterior of the plates, where the soil was visible, were covered with aluminium foil. Each plate was then placed horizontally in the growth chamber (16 h light period at 20°C). In order for the seedlings to develop, all plates were incubated for four weeks in the growth chamber prior to inoculations.

3.3.4 Treatments

The sterile microcosms were set up with four treatments of triple replicates, which includes seedlings with no inoculation (Control); inoculated with *Streptomyces* sp. A11; inoculated with *Heterobasidion annosum* and co-inoculated with *Streptomyces* sp. A11 and *H. annosum*. For each treatment, four identical sets of treatments were set up to harvest at different time periods (1-4 weeks post inoculation). Overall, 48 microcosms, totalling 144 seedlings, were included in this study.

Figure 1 A Microcosm set up in rectangle agar plate with three Pine seedlings growing inside.

3.3.5 Preparation of *Streptomyces* sp. Inoculum

Using a sterile stick, 100ml of liquid ISP2 medium was inoculated with *Streptomyces* sp. A11 from a colony grown on an agar plate (ISP2). The culture was then incubated on a shaker on 200RPM, at 28ºC for one week. Following the incubation, 50 ml of the

culture was moved to a falcon tube and centrifuged at 5000 RPM, at 20ºC for 20 minutes. The liquid was decanted and replaced by sterile water. Water was mixed with the hyphal sediments using cut sterile pipette tips. The tube was then centrifuged again. The liquid was decanted and replaced with 25 ml of sterile water. Water and hyphal sediments were mixed again. 1.05 ml of the mixture was pipetted into each plate (0.335µl per seedling). The liquid was pipetted on the soil around the seedlings with a slight distance from the roots.

3.3.6 Preparation of *H. annosum* inoculum

One millilitre of sterile water was added to malt extract agar plate containing freshly grown *H. annosum*. Using a sterile spreader, water was mixed with fungal hyphae on the plate. Mixed water (500 µl) was then pipetted into 250 ml of liquid malt extract media. The culture was incubated at 20 ºC for 7 days and was manually shaken once daily. Following the incubation, 50 ml of the culture was transferred to a falcon tube and was centrifuged at 5000 RPM, 20ºC for 20 min. The liquid was decanted and the fungal hyphae was weighed. Based on hyphal weight, about 25 ml of sterile water was added to obtain uniform concentration of *H. anossum* in each preparation. Water was mixed with hyphae using cut sterile pipette tips. 1.05ml of the mixed liquid was then pipetted into each plate (0.335µl per seedling). The liquid was pipetted directly on seedling roots in the soil.

For co-inoculation treatment, *Streptomyces* was first added, a week prior to *H. annosum* inoculation. For uniformity, all other samples (other treatments) were incubated for an extra 7 days. Additionally, other treatments received 1.05 ml of sterilised water instead of inoculum.

3.3.7 Pine Seedlings harvesting

Each set of samples were harvested at the appointed time after inoculation. Only two out of three seedlings in each plate were used for further examination. This was due to many of the plates having one dead or underdeveloped seedling. The Seedlings were then removed from the soil and were gently brushed to remove soil particles from the

root. Seedlings were weighed (fresh weight) and the number of lateral roots were measured. Seedlings (as a whole) were immediately frozen in liquid nitrogen, homogenised to powder using mortar and pestle and stored at -80°C. This was done to prevent RNA degradation in the seedlings as they were used in qPCR analysis.

3.3.8 Harvested Pine RNA extraction

RNA extraction was done following the method described by Chang et al. (1993). Concentration of RNA samples were tested using NanoDrop™ 2000/2000c Spectrophotometers (Thermo-Fisher Scientific).

3.3.9 Loss of RNA extracted samples

Unfortunately, due to human error and faulty material, the concentration of extracted seedling RNA of numerous samples were considerably low. Since the microcosms (with seedlings) would require a long period time to set up and maintain, and given the limited resources after the initial microcosm experiment, it was difficult to reproduce seedlings for gene expression analysis. Nevertheless, considerable efforts were exerted to synthesize complementary DNA (cDNA) from extracted RNA samples that had high enough quality and concentration. Overall, 11 Scots Pine defence related genes with 3 reference genes were tested on samples from different treatments that were harvested at different weeks (weeks 1-4). However, the use of several samples in real-time PCR was unsuccessful. As a result, gene expression analysis part of the project focused on *H. annosum* dual culture experiment.

3.4 Agar plate dual culture set up

3.4.1 Dual culture setup

Dual cultures of *H. annosum* (Isolate: 02034) and *Streptomyces* sp. A11 were set up on ISP2 agar plates. The surface of ISP2 agar plates were covered with a sterile

cellophane sheet, in order to facilitate the harvesting of fungal hyphae without interference by agar media. Diameter plugs (7 mm) were taken from the actively growing rims from both *H. annosum* and *Streptomyces* sp. A11 pure cultures, respectively. The plugs were set up with 45mm distance from each other and 20mm distance from the edge of the petri dish. Plates were then incubated in the dark at 25°C. For control, plates were set up with a *H. annosum* plug on one side and a plug from sterile ISP2 agar plate on the other side. Three sets of samples were set up to be harvested at different time points. The harvest time points were selected based on preliminary tests.

Each set of samples contained triple replicates and each replicate contained 6 individual plates in order to have sufficient amount of hyphae at the harvest. Similarly, three replicates for each time points were set up for control samples with only two plates for each replicate, since it was expected to have higher growth and more hyphae to be harvested. In total, the experiment included 54 plates of dual culture and 18 control plates were set up (Figure 2) Culture plates were also photographed at different time points to show progression of inhibition (see Figure 9).

Figure 2. An example of dual culture set up with *H. annosum* (right) and Streptomyces sp. A11 (left) (4 dpi).

3.4.2 Sample harvest and RNA extraction

The sets of samples were harvested at 9, 13 and 17 days post inoculation (dpi). *H. annosum* hyphal front was harvested from the side facing the *Streptomyces* in dual cultures. The harvested hyphae were immediately frozen using liquid nitrogen, homogenised using mortar and pestle and were stored at -80°C freezer. RNA extraction was done following a modified method of Chang et al. (1993). Concentration of RNA samples were quantified using NanoDrop™ 2000/2000c Spectrophotometers (Thermo-Fisher Scientific).

3.4.3 Primer design and selection

In order to have a better understanding of molecular basis for inhibition of growth of *H. annosum* cells, the expression of 10 genes from various cell functional groups were selected to be analysed. Primers for three of the mentioned genes were obtained from forest pathology lab (Baral et al. 2016, Raffaello et al. 2012). Primers for the other seven genes were designed using Roche Universal Probe Library Assay Design Center (https://lifescience.roche.com/en_fi/brands/universal-probe-library.html#assay-design-center).Transcript sequences for the designed primers were retrieved from JGI genome database (https://mycocosm.jgi.doe.gov/Hetan2/Hetan2.home.html; Olson et al. 2012). All primers used in this part of study are depicted in Table 1.

3.4.4 cDNA synthesis

The protocol for cDNA synthesis and qPCR were similar to the one used in Wen et al. (2019). Following the manufacturer's instruction (Thermo-Fisher Scientific), 1µg of total RNA was treated with DNase I (Sigma-Aldrich) prior to cDNA synthesis. Oligo(dT) primers were used in cDNA synthesis reaction. Reverse transcription of RNA was done by incubating the reaction mixer for 60 minutes at 42°C followed by heating the mixture for 5 min at 70°C in accordance to initial nucleic acid concentrations.

Table 1. List of all primers used in *H. annosum* qPCR experiment.

Gene name	5'-3'	Sequence	Putative function
Alpha Tub	Forward	TCCAGACGAACCTTGTACCC	Alpha Tubulin
	Reverse	ACAGAGTTCTGCTCGTGGTG	
RNA Pol3	Forward	AACAAGATGCGCTGGAAAG	RNA polymerase III transcription factor
	Reverse	GGAGCTCCTCACAATTTGGT	
Cytochrome P450	Forward	GGCACAAGCAGAGATTGACA	Stress related
	Reverse	TGTTCACATCGGAAAAGCTG	
Cyclophilin 1	Forward	AGACTGAGCGATGGTGACCT	Stress related
	Reverse	CTCACGTTGTCTTCGGTGAA	
Phosphoglucomutase (PGM)	Forward	ACTACGTCGAGCTCCTCCAG	Metabolism related
	Reverse	ACTACGTCGAGCTCCTCCAG	
Guanosine triphosphatase (GTPase)	Forward	GTGGTCACGTCAGCTTTGAA	Signalling related
	Reverse	TGTAACGCGCCAGAGTATTG	
hydrophobin 2	Forward	CGTCGCTCGTGATACCATC	Defence/virulence
	Reverse	TTGCAGCACTGCAGATCAC	
glutamine synthetase	Forward	GTAGGGAGTGCCATCGACAT	Metabolism related
	Reverse	GGACTCCGTCCCTTGGTT	
HaHOG1	Forward	ATGTCTTTCGTCAAGCTCAG	Signalling (MAPKs)
	Reverse	CTAGCTACAACAGGCC	
TSL1	Forward	CGAAATGCAACAGAAGGTGAT	glycosyltransferase family
	Reverse	CGAGAAGCGCGAGTTGAT	
ABCG1.1(66124)	Forward	GCCATCTTGTGTACCATCCA	ABC transporter family
	Reverse	TCGCCAAAGTAGACTGTCCTG	
hassp30	Forward	ACTTAGCGAACATCGTGATCC	Small secreted protein
	Reverse	TGCCTGTGCCTTTTTGATCT	

3.4.5 Quantitative real-time PCR

5µl of cDNA was diluted in 95µl of nuclease free water (NFW). In addition to cDNA template, qPCR reaction mixture was prepared. The qPCR reaction mixture consisted of 1µl of forward primer, 1µl of reverse primer and 7.5µl of 2X LightCycler 480 SYBR Green I Master (Roche, Finland). The quantitative real-time PCR program was as follows: 5 minutes denaturation at 95℃, 45 amplification cycles of 10 second at 95℃, 10 second annealing at 60℃ and extension 20 second at 72℃.

The experiment was conducted with three biological replicates and two technical replicates. The relative quantities of genes used in the experiment were normalised using two reference genes, RNA Polymerase 3 and Alpha Tubulin (Table 1).

3.5 Optical microscopy observations

Separate dual culture plates and control plates were set up and incubated following same protocol as mentioned earlier (see chapter 3.6.1). *H. annosum* hyphae and *Streptomyces* sp. A11 Hyphae were extracted from an actively growing tip of the culture, placed on cover slips and observed using Olympus CX31 binocular microscope. Pictures were captured with Olympus SC30 microscope camera using the cellSense software. Observations were done at 5, 9 and 14 days post culture setups in order to observe potential morphological changes before visible inhibition in dual culture, start of clear inhibition of *H. annosum* by *Streptomyces* sp. A11 and when fungal growth slowed down drastically. For both *H. annosum* and *Streptomyces* samples were taken from dual cultures (*H. annosum* & *Streptomyces* sp.) and pure cultures of each isolate (control).

3.6 Statistical analysis

For strain selection test, the data was presented as means (±SD). Shapiro–Wilk's test for normality was used to check for normal distribution. Data from three strains of *Streptomyces* sp. for each growth media was tested independently using one-way analysis of variance (ANOVA) test.

Seedling fresh weight data was presented as means (±SD). Lateral root formation data was presented as means (±SE). In both data sets, for each set of data (1-4 week), Shapiro–Wilk's test for normal distribution was used. When data was normally distributed ($P>0.05$), one-way ANOVA test was performed to determine whether there is significant difference between two or more treatments. If so ($P<0.05$), Tukey's HSD post hoc test was used to determine which treatments have significantly different values. If the Shapiro–Wilk test showed that the data was not normally distributed ($P<0.05$), $\log_{10}$ transformation efforts were made to normalise the data. when $\log_{10}$ transformation did not normalise the data, Kruskal-Wallis test was used to determine if there is any significant difference between two or more treatments.

For quantitative real time PCR study, the mean of data output (Ct values) of genes were used to calculate fold change of selected genes. Results from control samples

from each time point (9, 13, 17 dpi), were used against other samples of that time frame by applying $\Delta\Delta C_T$ method. Additionally, reference genes data was used to calculate relative quantifications (BIO-RAD, 2005).

Normalised data of each gene and in each time frame, was tested for normality followed by test for significant difference compared to appropriate control data for that gene at that time point. Test for normality of data distribution was done using Shapiro–Wilk's test. Normally distributed data was then tested for significant difference using independent t-test. Data sets that were not normally distributed were tested for significant difference using Mann–Whitney U test.

All the statistical analysis tests were done using SPSS program (IBM SPSS Statistics version 24.0).

4. Results

4.1 Strain selection dual culture setup

In all the agar media tested, *Streptomyces* sp. A11 strain had significantly higher zone of inhibition against *H. annosum* in comparison to one or both of the other tested *Streptomyces* strains. (Figure 3).

In Malt extract agar culture, both A11 and FY4 strains had significantly higher inhibition zone in comparison to B2 strain (P=0.00, P=0.00). In MMN culture, A11 strain had significantly higher zone of inhibition compared to FY4 and B2 strains (P=0.001, P=0.042). Results from ISP2 medium showed that A11 has significantly higher zone of inhibition compared to FY4 and B2 (P=0.003, P=0.00). In Hagem agar, A11 showed significantly higher zone of inhibition compared to FY4 strain (P=0.00). Based on the provided results, and observations of plates through the incubation stages, *Streptomyces* sp. A11 was selected and used for the rest of the experiment.

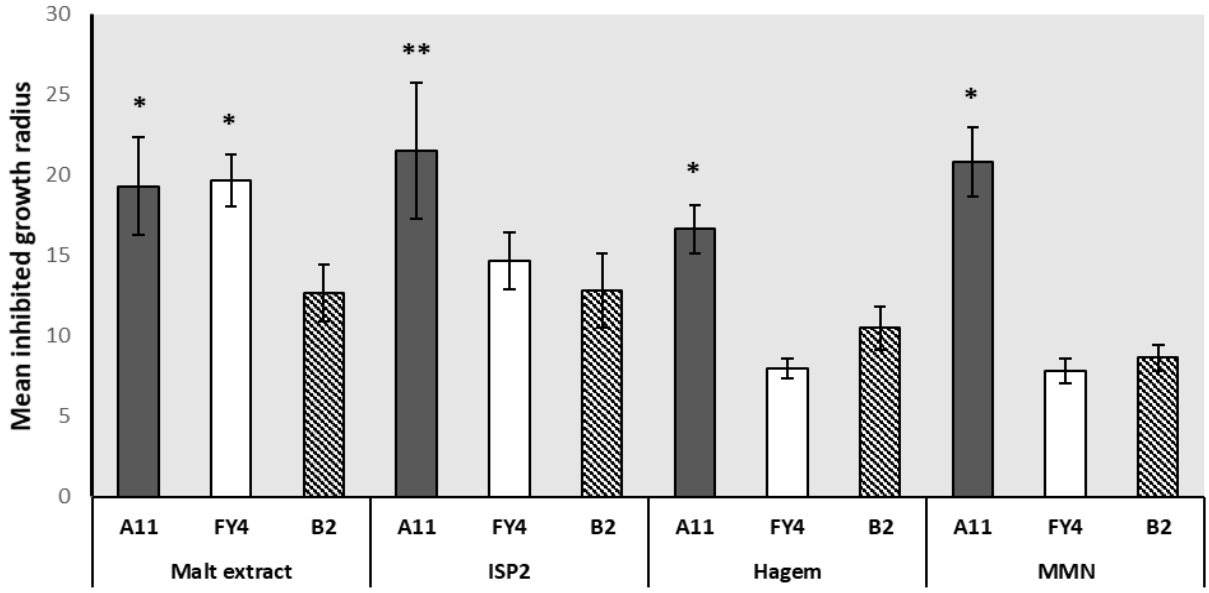

Figure 3. Inhibition radius of *H. annosum* against *Streptomyces* strains A11 (dark grey), FY4 (White) and B2 (Pattern) tested in four different agar mediums. Asterisks on top of the bars indicate significantly higher inhabitation radius of that strain (within that media), compared to one other strain (one asterisks) or both other strains (two asterisks). Error bars indicate standard deviation from 6 biological replicates for each treatment.

4.2 Plant fresh weight

Week 1

Fresh weight data of harvested seedlings from week 1 were normally distributed (P=0.92)

Further tests displayed a significant difference between treatments (P=0.04). *H. annosum* treatment has significantly lower fresh weight than co-inoculation treatment (P=0.044) (Figure 4).

Week 2

Fresh weight data of harvested seedlings from week 2 were normally distributed (P=0.48). Further tests illustrate that there was a significant difference between two or more treatments in second week (P=0.00). Fresh weight of co-inoculation, pathogen and *Streptomyces* sp. A11 treatments were all significantly lower than control treatment (P=0.00, P=0.00, P=0.027 respectively). Additionally, *Streptomyces* sp. A11 treatment was significantly higher than co-inoculation and *H. annosum* treatments (P=0.03, P=0.005 respectively). There was no significant difference between *H. annosum* and co-inoculation treatment (P=0.996) (Figure 4).

Week 3

Fresh weight data of seedlings from week 3 was normally distributed (P=0.92). There was a significant difference between two or more of the treatments (P=0.09). Results from further tests demonstrated that fresh weight of seedlings from co-inoculation treatment was significantly lower than the ones from control treatment (P=0.011). Additionally, Co-inoculation treatment had significantly lower fresh weight compared to *H. annosum* treatment (P=0.034) (Figure 4).

Week 4

Fresh weight data of seedlings from week 4, was normally distributed (P=0.851). There was a significant difference between two or more of the treatments (P=0.04).

Fresh weight of co-inoculation treatment was significantly lower than control treatment (P=0.002) (Figure 4).

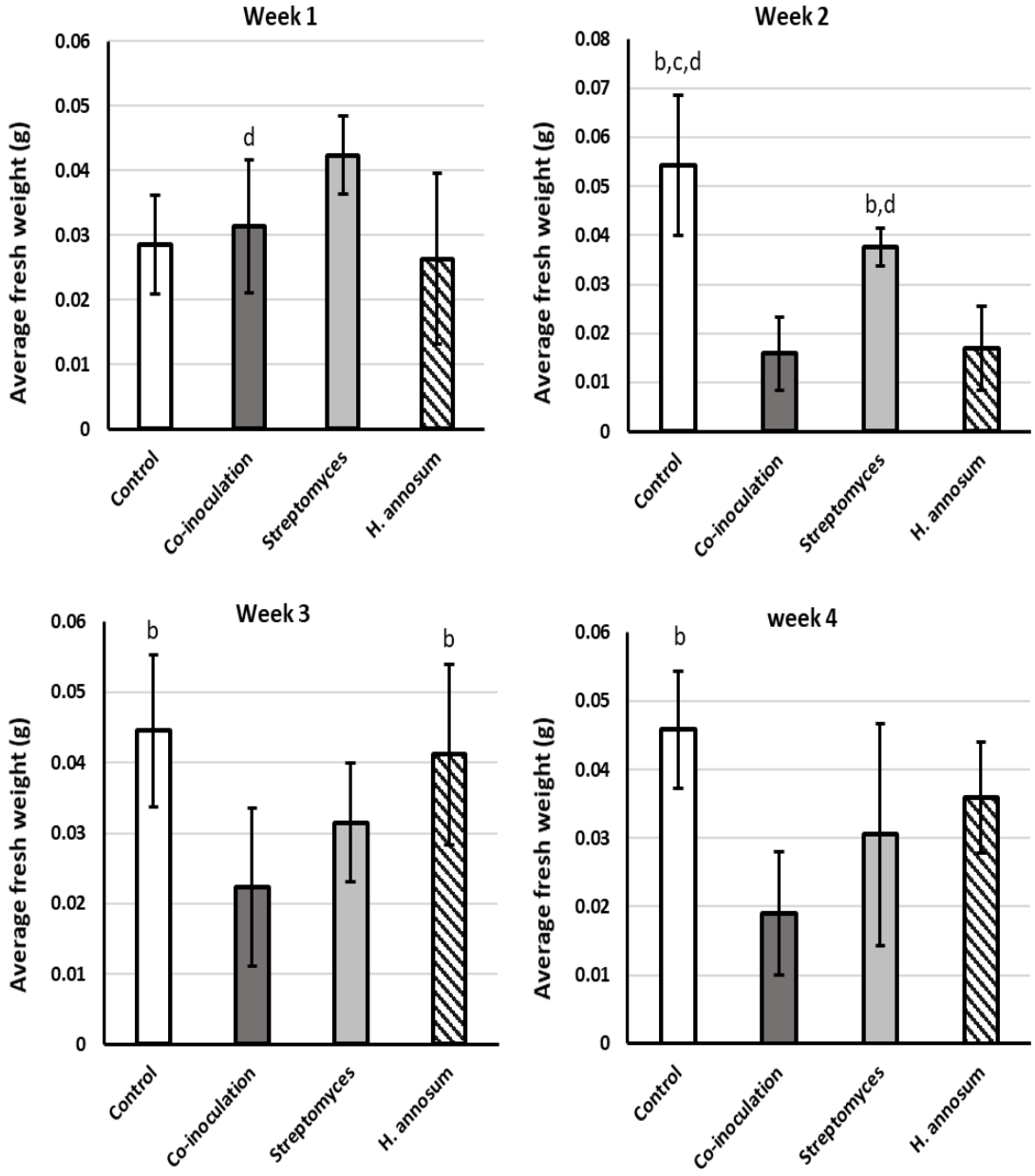

Figure 4. Average fresh weight of seedlings harvested at four different time points (1-4 weeks post inoculation). Error bars represent standard deviation from 6 biological replicates. Common letters above bars indicate significantly higher values compared to Control (a), Co-inoculation (b), Streptomyces sp. A11 (c) and H. annosum (d) treatment.

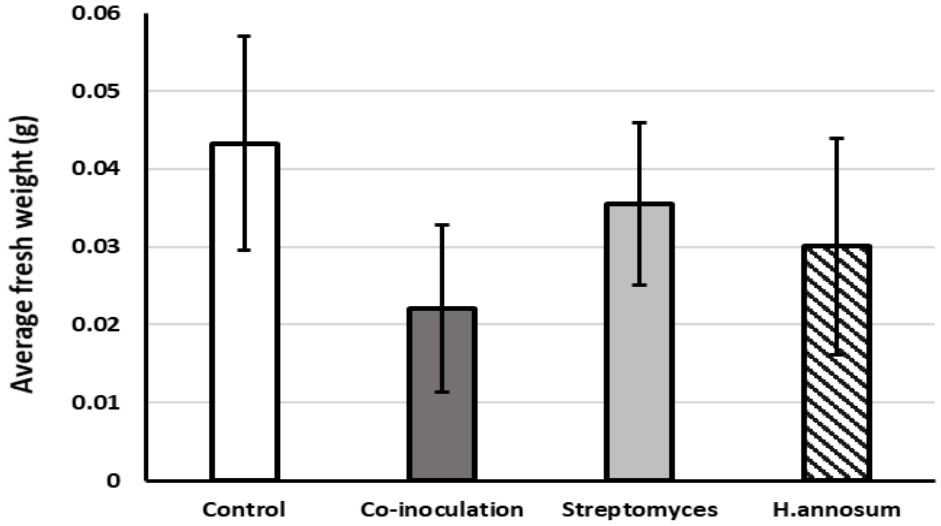

Figure 5. Overall average fresh weight of all harvested seedlings throughout the 4 weeks of harvesting. Error bars represent standard deviation from 24 biological replicates.

4.3 Seedling lateral root formation

Week 1

Lateral roots data of harvested seedlings from week 1 was not normally distributed (P=0.07). Log_{10} transformed data, remained not normally distributed (P=0.007). Results from further tests demonstrated that there was no significant difference between treatments in first week (P=0.162) (Figure 6).

Week 2

lateral roots data of harvested seedlings from week 2 was not normally distributed (P=0.00). Log_{10} transformation of the data did not normalise it (P=0.002). Results from further tests demonstrated that there was significant difference between two or more treatments (P=0.001). Further analysis showed that both Co-inoculation and *H. annosum* treatments were significantly lower than control treatment (P=0.004, P=0.003 respectively) (Figure 6).

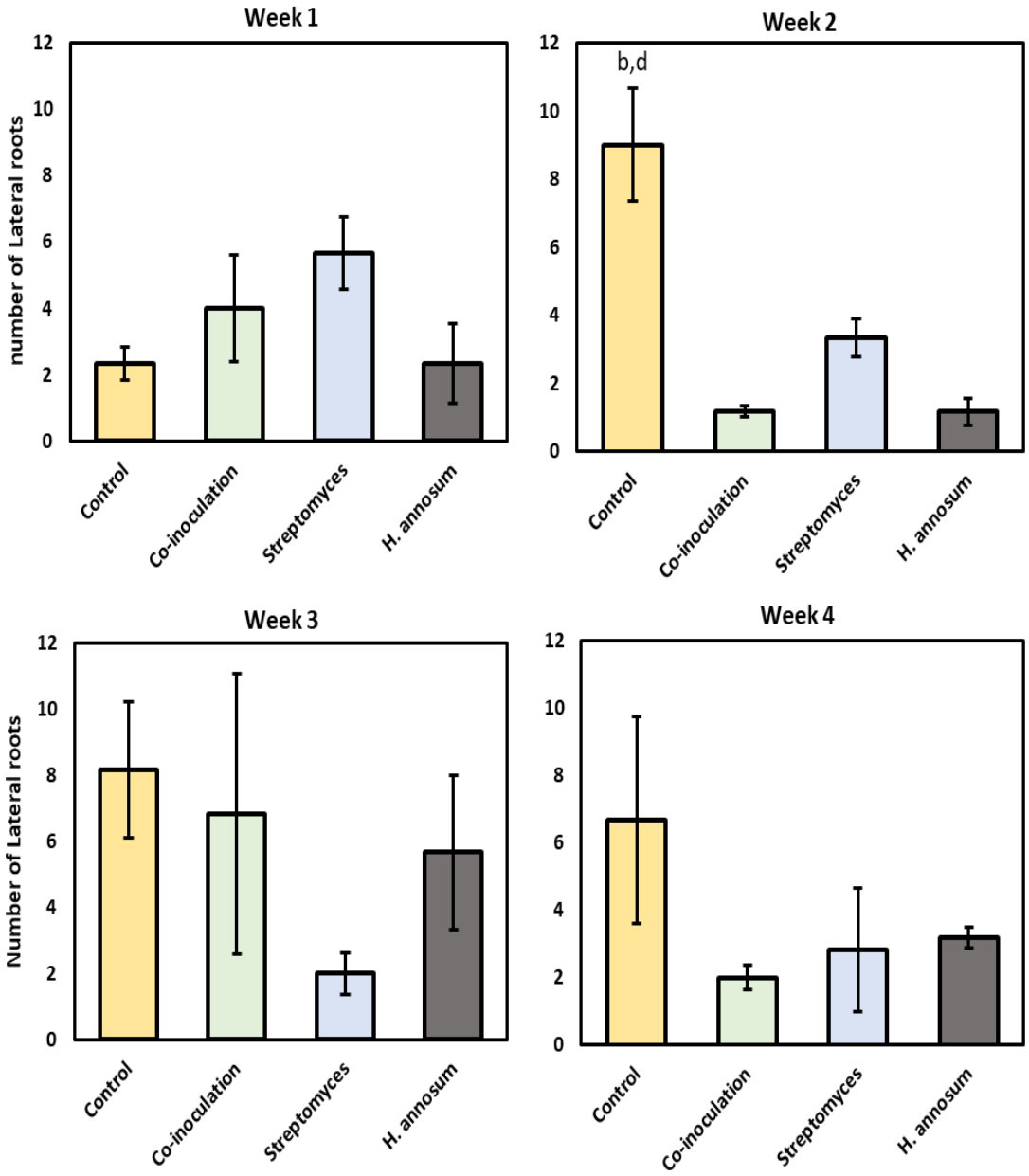

Figure 6 Average number of lateral roots of Seedlings, harvested at four different time points (1-4 weeks post inoculation). Error bars represent standard error from 6 biological replicates. Common letters above bars indicate significantly higher values compared to Control (a), Co-inoculation (b), Streptomyces (c) and H. annosum (d) treatment.

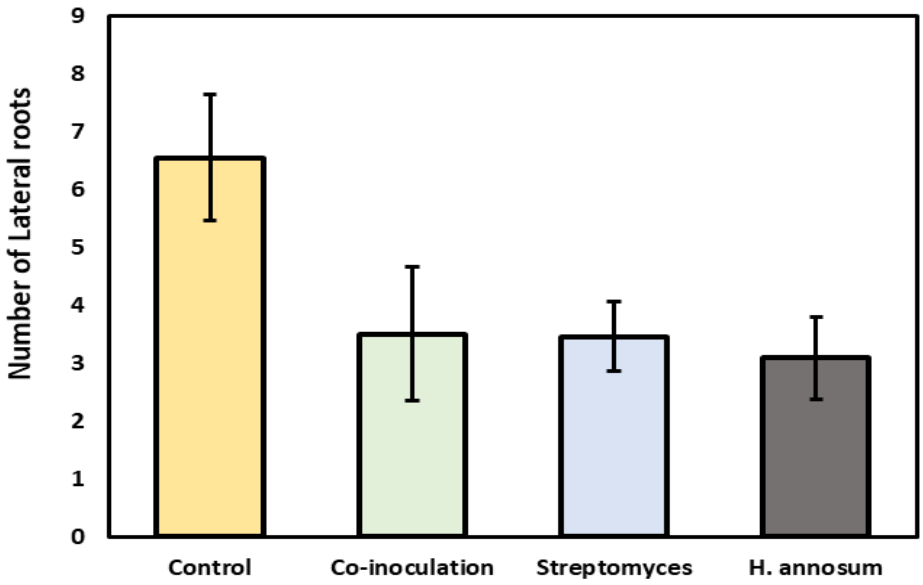

Figure 7. Overall average lateral root formation of all harvested seedlings throughout the 4 weeks of harvesting. Error bars represent standard error from 24 biological replicates.

Week 3

Data for week three of seedling's lateral roots formation was not normally distributed (P=0.00). $\log_{10}$ transformation of the data did normalise it (P=0.144). Further tests showed that there was no significant difference between lateral roots in treatments in week 3 (P=0.16) (Figure 6).

Week 4

Lateral roots formation data from harvested seedlings from week four, was not normally distributed (P=0.00). $\log_{10}$ transformation of the data did not normalise it (P=0.008). Further tests illustrated that there was no significant difference between treatments in week 4 lateral roots data (P=0.084) (Figure 6).

4.4 *H. annosum* gene expression in response to dual interaction with *Streptomyces* sp. A11

Out of the 10 target genes of *H. annosum*, only 5 showed reproducible expressions. The calculated fold change of these genes depicted in Figure 8.

4.4.1 Hydrophobin 2

Expression levels of Hydrophobin 2 gene (JGI protein ID: 148119) in *H. annosum* under dual culture revealed that the gene was initially upregulated at 9 and 13 dpi, having the highest induction at 9 dpi followed by down-regulation at 17 dpi compared to control treatments. Normalised expression fold data at 17 dpi was significantly lower compared to relative control treatment (P=0.00) (Figure 8).

4.4.2 Cytochrome P450

Expression levels of Cytochrome P450 gene (NCBI accession no. AY827553) was upregulated in all three time points compared to control treatments. Highest level of Cytochrome P450 induction was at 9 dpi (Figure 8).

4.4.3 Guanosine triphosphatase (GTPase)

GTPase gene (NCBI accession no. BU672418) was upregulated at all three time points compared to control treatments. Highest gene induction level was at 13 dpi followed by 9 and 17 dpi. Normalised expression fold data at 17 dpi was significantly higher than control treatment (P=0.037) (Figure 8).

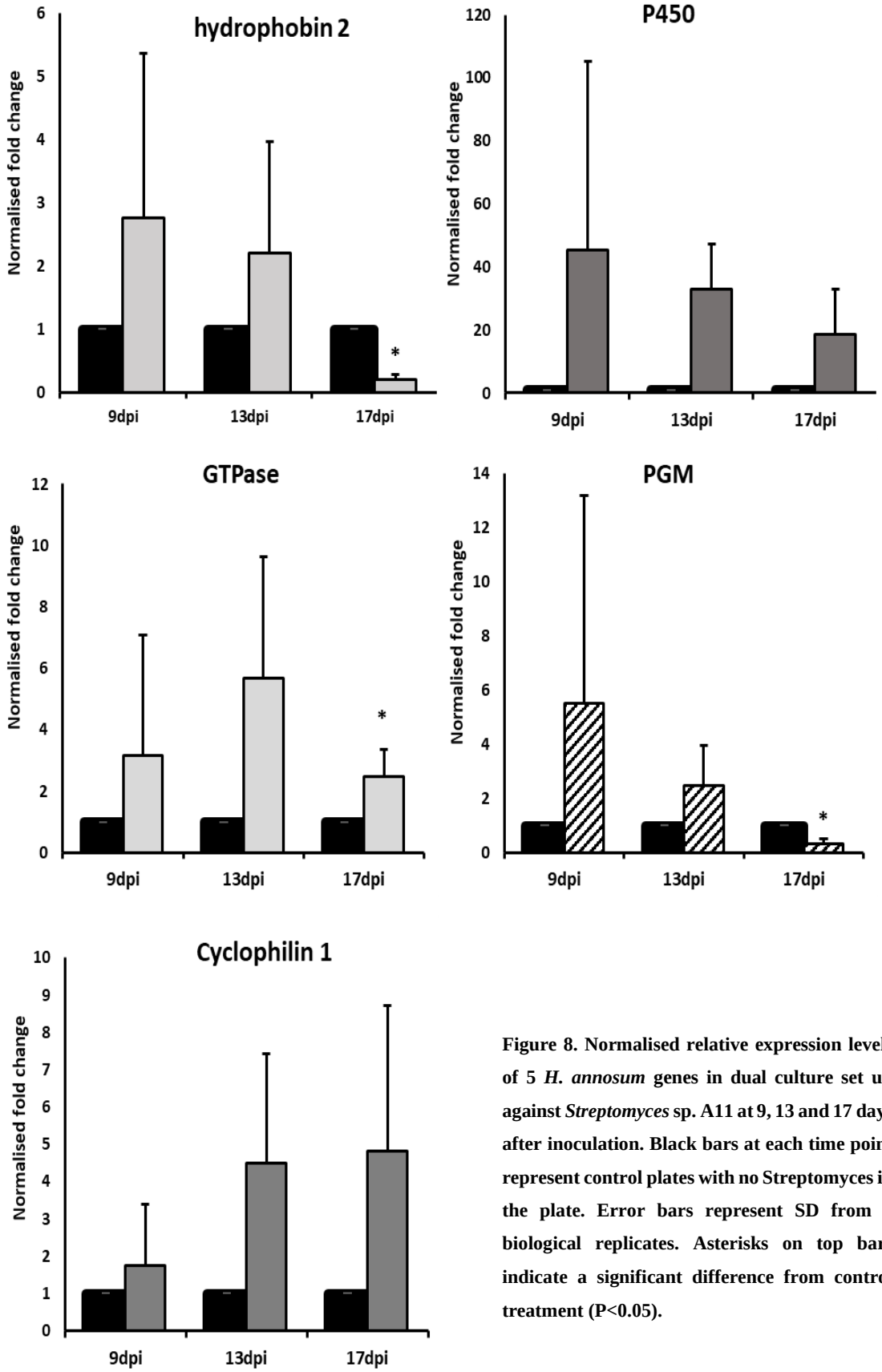

Figure 8. Normalised relative expression levels of 5 *H. annosum* genes in dual culture set up against *Streptomyces* sp. A11 at 9, 13 and 17 days after inoculation. Black bars at each time point represent control plates with no Streptomyces in the plate. Error bars represent SD from 3 biological replicates. Asterisks on top bars indicate a significant difference from control treatment (P<0.05).

4.4.4 Phosphoglucomutase (PGM)

Expression levels of PGM gene (NCBI accession no. CK817377) was upregulated at the first two time points and then downregulated in 17 dpi, compared to control treatments. Having the highest gene induction at 9 dpi followed by 13 dpi. Normalised expression fold data at 17 dpi was significantly lower compared to relative control treatment (P=0.037) (Figure 8).

4.4.5 Cyclophilin 1

Expression levels of Cyclophilin 1 gene (NCBI accession no. FJ898366) was upregulated at all three time points with the gene induction gradually increasing as time went by compared to control treatments. Cyclophilin 1 was induced the highest at 17 dpi (Figure 8).

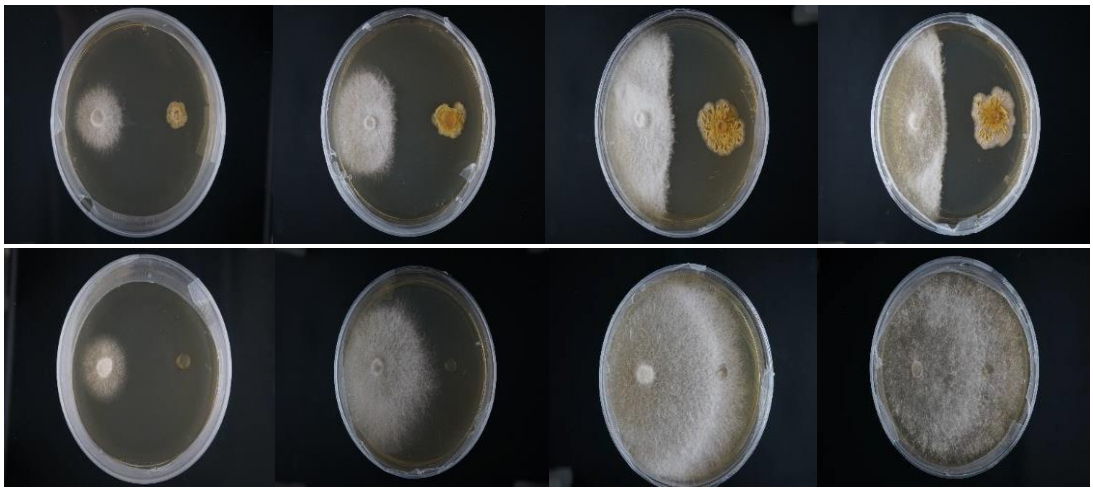

Figure 9. Progression of Dual culture days after the set up. Top Row: Dual culture with *H. annosum* (Left plug) and *Streptomyces* sp. A11 (Right plug) at 4,9,13 and 17 dpi. Bottom row: Control set up with *H. annosum* (Left plug) and sterile plug (right plug) in 4,9,13 and 17 dpi.

4.5 Optical microscopy observations

4.5.1 *Streptomyces* sp. A11

Streptomyces sp. A11 was observed at three different time points (5, 9, 14 dpi) in dual culture conditions against *H. annosum* and in monoculture as control. Overall, no visible morphological changes were observed between *Streptomyces* sp. A11 in control and dual culture setup. Moreover, samples observed over three time points did not indicate any visible change (Figure 10).

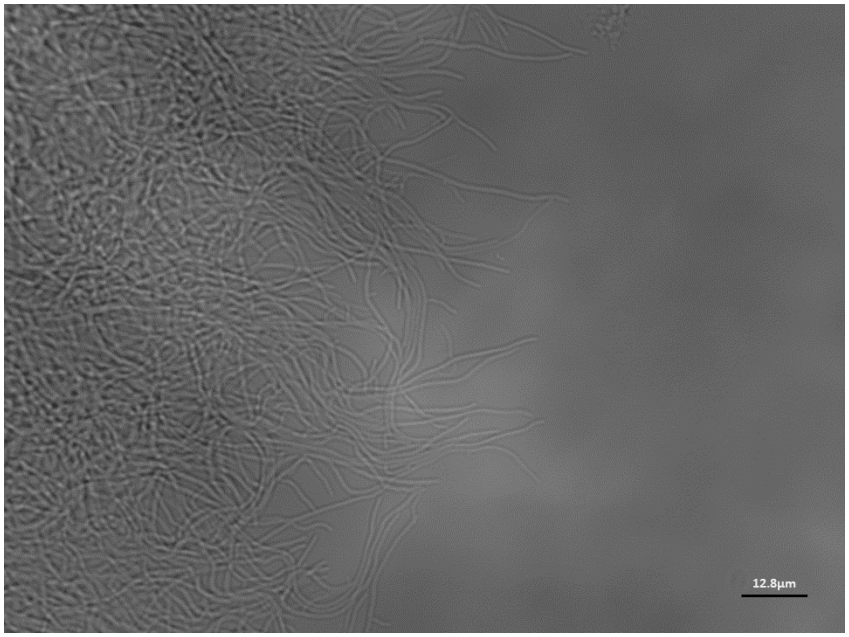

Figure 10. Microscopic images of *Streptomyces* from dual culture. Hyphal-like growth of actinobacteria is visible in these pictures.

4.5.2 H. annosum

H. annosum was observed in three different time points (5, 9, 14 dpi) in dual culture conditions against *Streptomyces* sp. A11 and in pure culture as control. Indication of *H. annosum* hyphae being under stress was observed in all three sampled time points including in the first time point (5 dpi) samples prior to visible (i.e., naked eye) growth inhibition (Figure 11_c).

Compared to the control samples, some of the fungal hyphae were enlarged and displayed large, visible vacuoles (Figure 11_d). Additionally, some of the fungal hyphae appeared to be transparent suggesting that the hyphae had undergone osmotic lysis and thus, the cytoplasm and organelles have leaked out from the cell. Another observation that was apparent from 9 and 14 dpi was the increased hyphal branching on the edge of the culture compared to their relative control samples (Figure 11_e, f).

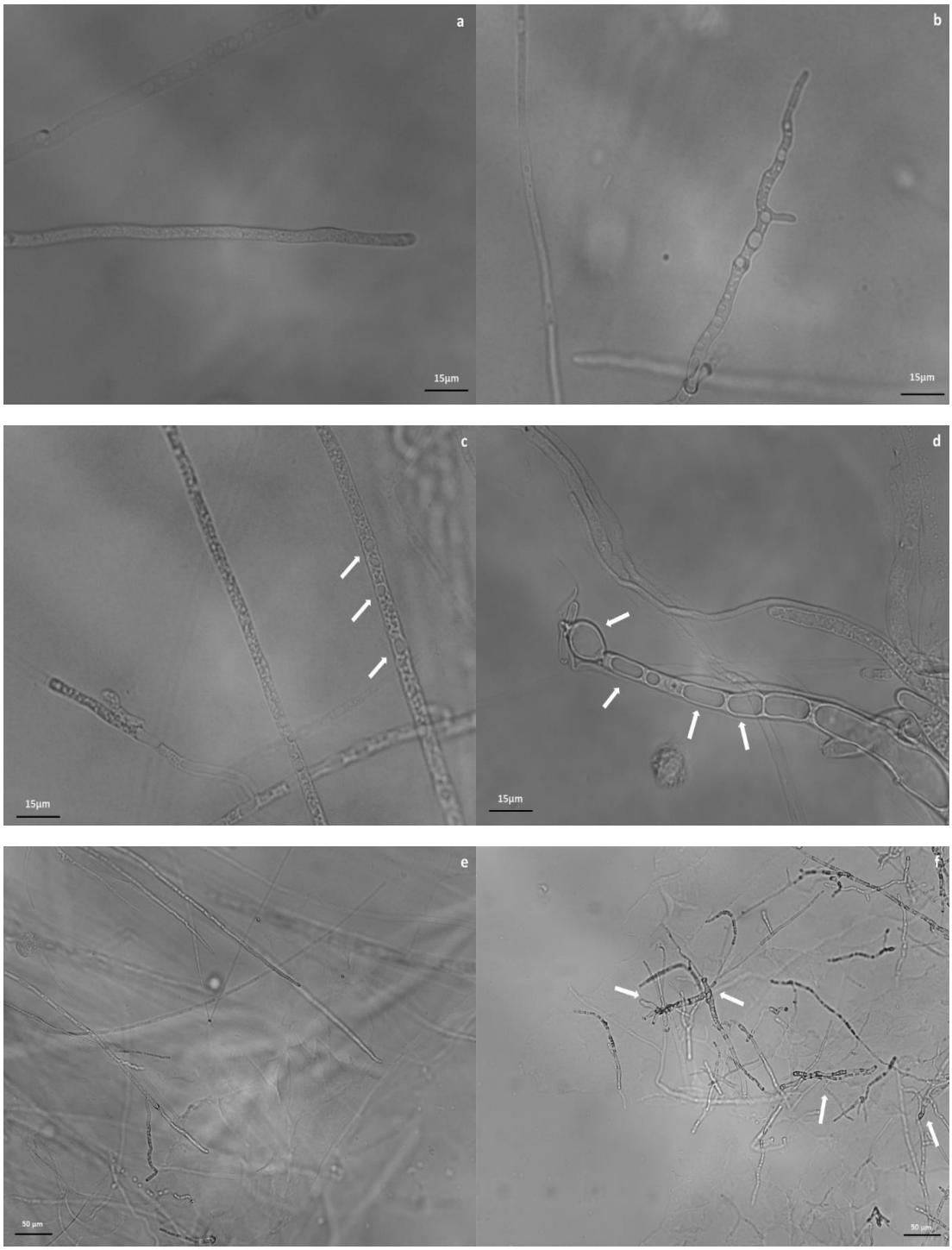

Figure 11. a-f) Microscopic images of *H. annosum.* a-b) images of healthy hyphae tip under control conditions at 9 (a) and 14 (b) dpi. c) Hyphae from dual culture treatment sampled at 5 dpi. Arrows indicate the visible high number of vesicles and some vacuoles in the hyphae. d) *H. annosum* hyphae from dual culture treatment sampled at 14 dpi. Arrows indicate the enlarged hyphae tip and enlarged vacuoles. e) Hyphae samples from 9 dpi control treatment. Visible hyphae appear to be growing in a straight line and parallel amongst other growing hyphae. f) Hyphae samples from 9 dpi dual culture treatment. Arrows indicate the highly branched and malformed formation of hyphae compared to control (e).

5. Discussion

This study evaluated how a potentially novel strain of Streptomyces bacteria associated with mycorrhizal fungi, interacts with pine seedling and one of pine's major pathogenic fungi, *H. annosum*.

From preliminary and other observations, it became clear that *Streptomyces* sp. A11 can adeptly supress the growth of *H. annosum* in agar media culture. However, the effects and interactions of *Streptomyces* sp. A11 on *H. annosum* appeared to be more complex when pine seedlings were present in the microcosm.

5.1 Plant fresh weight

The analysed data from each week of sampling, showed no difference between treatments that is significant in all 4 weeks. Nevertheless, other observations are established from the results.

Seedlings treated with *Streptomyces* sp. A11 did not have significantly higher fresh weight compared to control seedlings in any of the time points. Thus, from the first hypothesis, we can reject that *Streptomyces* sp. A11 alone can improve overall growth of pine seedlings. This in accordance with Golinska and Dahm (2013) study where pine seedlings inoculated with isolated *Streptomyces* sp. alone had small positive effects on the plant. Studies portraying, the positive effects of Streptomyces inoculation on plants are mainly performed under stress conditions where plants are grown under abiotic stress or in presence of pathogens (Sadeghi et al. 2012; Palaniyandi et al. 2014; Rey and Dumas 2017). Hence, comparing seedlings from *Streptomyces* inoculated treatment with seedlings grown in sterile soil may not reveal the full interactive effects of *Streptomyces* sp. A11.

Seedlings from co-inoculation treatment had significantly lower fresh weight compared to seedlings inoculated only with *H. annosum* in the third week. Similarly, this disparity can also be observed in the overall average of the seedling's fresh weight (Figure 5). These observations indicate that co-inoculation of seedlings with both the bacteria and the pathogenic fungi did not benefit the seedlings in mitigating *H.*

annosum infection, and conversely, it may have caused more severe adverse effects compared to seedlings infected by the pathogen alone. Therefore, we can reject the second hypothesis since the data indicate the opposite effects. This is in accordance with certain samples in Lehr et al. (2007) study (chapter 1.4), where the only *Heterobasidion* sp. isolate tolerant to *Streptomyces* sp. inhibition, was also able to have improved colonisation of spruce because of inhibition of plant defence response by the *Streptomyces* species. Although in our study, *Streptomyces* sp. A11 was able to supress *H. annosum* growth in dual culture setup, it is plausible that *Streptomyces* sp. A11 also suppressed the immune response of Pine seedlings in co-inoculation treatments. Another possible reason is that competition for nutrients, within the soil of the microcosm, between all three species (plant, bacteria and fungi) in the co-inoculation treatment, may have placed the pine seedlings at a disadvantage for nutrient intake. Consequently, it resulted in lower growth of seedlings even in comparison to microcosms with only seedlings and *H. annosum* present.

Despite the current results, *Streptomyces* sp. A11 may have a different response to plant and pathogenic fungi if inoculated along with mycorrhizal fungi since the MHB *Streptomyces* have a close relationship with mycorrhizal fungi and their symbiosis formation with the plant (Schrey and Tarkka 2008). Moreover, studies done by Golinska and Dahm (2013) showed that positive effects of *Streptomyces* isolates on pine were higher in non-sterile soil compared to sterile one. This further suggests that *Streptomyces* sp. A11 may have different effects on infection of pine seedlings by *H. annosum* or other plant pathogens, if additional microbes or microbe communities are present in the soil.

Presence of soil in the microcosm may have influenced the behaviour of both *Streptomyces* sp. A11 and *H. annosum.* Both species are naturally occurring in forest soils and both can produce hyphae, which can be advantageous in the soil environment. To the best of author's knowledge, there has only been on study (Golinska and Dahm 2013) examining all three similar components (tree seedling, actinobacteria and pathogenic fungi) in microcosms with soil present rather than in test tubes or agar plates (see chapter 1.4).

5.2 Lateral root formation

Seedlings treated with *Streptomyces* sp. A11 did not show significantly different number of lateral roots compared to control seedlings in any of the time points. Hence, from the first hypothesis, we can also reject that *Streptomyces* sp. A11 inoculation alone can improve root development of pine seedlings. This result is similar to a study from University of Tokyo (unpublished data) using the exact same *Streptomyces* isolate (A11). The aforementioned study showed that the A11 strain could quickly affect mycorrhizal fungal growth and promote mycorrhizal formation in the early stage. However, the effect on plant root was very limited. Based on this and fresh weight data, we can argue that although, *Streptomyeces* sp. A11 inoculation alone, did not proved to be beneficial for pine seedlings in growth and root development, it did not have overall significant deleterious effects on seedlings.

In the second week, seedlings from *H. annosum* and co-inoculated treatments both had a significantly lower number of formed lateral roots in comparison to the control treatment. This is relative to fresh weight results of mentioned treatments and may result from overall stunted root and shoot development in the seedlings of those treatments.

Increase in the number of plant's lateral root induced by *Streptomyces* sp. may not always be beneficial. Some pathogenic *Streptomyces* sp. are shown to produce Nitric oxide (NO) when in contact with target host plants. NO can cause signalling and physiological changes in plants including increase in lateral root formation (Neill et al. 2003; Johnson et al. 2008). Increase in lateral root formation also increases available infection sites. Alternatively, some plant benificial *Streptomyce* sp. have been observed to significantly increase plant biomass and number of lateral roots when grown under salt stress conditions (Palaniyandi et al. 2014).

The results from lateral root data was less conclusive, as most of the data showed no significant difference in lateral root formation of seedlings. Based on observations during harvesting the samples, seedlings extracted from same rectangle plate (i.e., same environment) and having seemingly similar shoot size, showed very high variation in the number of formed lateral roots. This may be due to high variance in seedling phenotypes and the small sample size.

5.3 *H. annosum* gene expression in antagonism

Based on *H. annosum* gene expression results, we can accept the third hypothesis and state that *H. annosum* grown in a dual culture with *Streptomyces* sp. A11 does have differentially expressed genes compared to monocultures.

5.3.1 Hydrophobins

The initial upregulation of the targeted hydrophobin gene can be in response to stress caused by *Streptomyces* sp. secondary metabolites and an attempt by *H. annosum* to self-regulate against it. In the later time point, Hydrophobin gene had lower upregulation levels followed by significant down-regulation at 17 dpi samples. This suggests that the secondary metabolites produced by Streptomyces have ultimately supressed the hydrophobin gene expression to supress the fungal growth and survivability.

Hydrophobins are strong surface-active proteins with a variety of roles in different stages of fungal growth such as structural functions, defence related, survivability and adaptation to the environment via surface interaction (Linder et al. 2005; Adomas et al. 2006). Hydrophobin gene studied in this experiment is suggested to have a role as structural component of fungal cell wall (Mgbeahuruike et al. 2013).

Similar findings were found in Mgbeahuruike et al. (2013) study, where culture filtrate of an antagonistic fungi *Phlebiopsis gigantea* was used against *H. annosum.*

5.3.2 Cytochrome P450

The upregulation of targeted Cytochrome P450 gene at all three time points compared to their respective controls, although not significant, can be due to stress response of

H. annosum to potential antifungal compound released to the medium by *streptomyces* sp. A11

Cytochrome P450 mono-oxygenases belong to a super family of proteins with many potential functions such as growth, metabolism and detoxification of xenobiotic compounds. White rot fungi (e.g., *Heterobasidion* sp.*)* have seemingly high number of genes related to Cytochrome P450 family. Therefore, studies suggest that Cytochrome P450 genes may have a role in detoxifying antifungal compounds released from degradation of wood (Yadav et al. 2006; Nelson et al. 2004).

In the study of gene expression of *H. annosum* against *Streptomyces* derived antifungal compounds, Lehr et al. (2009) also observed an upregulation in Cytochrome P450 gene after exposure to the antifungal compound. Interestingly, Lehr et al. (2009) observed that gene expression of cytochrome P450 in *H. annosum* was highly dependent on concentration and length of exposure to the antifungal compound: P450 gene was initially upregulated but then downregulated in later time points (0.5h, 2h, 6h). This is similar to results of this study, where upregulation of P450 gene was reduced (but not downregulated) over time. It can be hypothesised that expression of Cytochrome P450 genes in *H. annosum* are highly dependent on the length and concentration of exposure to antifungal compounds.

The specific P450 gene used in this work, was studied by Karlsson et al. (2008) whereby, it was shown that the *H. annosum* P450 gene was upregulated when grown on the bark of living spruce tree or on low nitrogen medium.

5.3.3 GTPase

Expression levels of targeted GTPase gene was upregulated overall, with significant upregulation at 17 dpi. Possibly, the *H. annosum* hyphae changed the direction of their growth due to inhibition by potential Streptomyces derived compounds.

GTPases are class of proteins that have roles in diverse aspects of regulating morphology of eukaryotic cells by mediating vesicle transport in different parts of the cell (Labbaoui et al. 2017). GTPase signalling proteins were shown to have a key role

in fungal filamentous hyphal growth and re-orientation of tip growth in response to the environmental factors (Brand and Gow 2009).

The specific GTPase gene used in this study was previously used by Lehr et al. (2009) mentioned previously. Similar to our results, the *H. annosum* GTPase gene was upregulated in response to antifungal compound produced by *Streptomyces*.

5.3.4 Phosphoglucomutase (PGM)

Targeted PGM gene showed the highest upregulation at first time point (9 dpi), followed by decreased upregulation and eventually (17 dpi), a significant down-regulation compared to control samples.

Phosphoglucomutases (PGM) are enzymes involved in glucose metabolism (Mgbeahuruike et al. 2013). PGM enzymes have a role in glucose and sucrose biosynthesis as well as trehalose (one of the main forms of stored sugar in fungi) anabolism (Li et al. 2006).

The findings of early upregulation of targeted PGM gene is similar to those of Lehr et al. (2009), that found significant upregulation in PGM genes of both *H. annosum* and *H. abietinum*, 2h after treatment with Streptomyces derived antifungal compound. This suggests that overall stress caused by *streptomyces* sp. A11 antifungal compounds can induce *H. annosum* PGM gene transcripts.

The specific gene used in this study was also used by Adomas et al. (2006). In that study, dual culture of antagonistic fungi species *Phlebiopsis gigantea* and *Heterobasidion parviporum* were setup and gene expression of both species were tested at different conditions. Results from Northern blot analysis showed a high expression of PGM in both species throughout the experiment. Although, at early and later stages of the experiment (9 and 28 dpi), there was a notable downregulation in PGM gene. The downregulation of PGM in *H. parviporum* in later stage of Adomas et al. (2006) study, could be due to similar causes as in this study.

Mgbeahuruike et al. (2013) observed downregulation of PGM genes in *H. annosum* after exposure to culture filtrate from antagonistic fungi *P. gigantean*. Thus, suggesting that certain secondary metabolites produced by antagonistic species to *H. annosum*

directly suppress PGM gene induction and as a result disrupt glucose metabolism in *H. annosum* cells.

Although Mgbeahuruike et al. (2013) study was focused on H. *parviporum* rather than H. *annosum*, study suggests that cDNA arrays of one intersterility group (e.g., H. annosum), can be used to study gene expression in the other group (e.g. H. *parviporum*), due to high correlation in sequence homolog and gene expression levels between the groups (Lundén et al. (2008; Lehr et al. 2009).

5.3.5 Cyclophilin 1

In this study, Cyclophilin 1 was upregulated at all time points with a gradual increase in upregulation from 9 dpi to 17 dpi. There was only minimal increase from 13 dpi to 17 dpi. This indicates a response of *H. annosum* to cell stress caused by potential antifungal compounds produced by *Streptomyces* sp. A11. The small increase in upregulation from 13 dpi to 17 dpi correlates with hyphal growth of *H. annosum* during those time points in the dual culture. Hyphal growth speed is slowed down from about 13 dpi onwards and *H. annosum* hyphae seem to form thickened mycelium on the edges (Figure 9).

Cyclophilins are a group of highly conserved proteins that have a regulatory role in many cellular processes such as cell cycle control, calcium signalling transcriptional repression and response to environmental stress. They have also been found to be a virulence factor in certain fungal pathogens (Viaud et al.2002; Adomas et al. 2006).

It is difficult to interpret upregulation in the Cyclophilin 1. For example, Schrey et al. (2005) study found upregulation in different Cyclophilin gene in non-pathogenic, ectomycorrhizal fungus *A muscaria* that was promoted by *Streptomyces* sp. in similar setups. In that study, it was suggested that growth stimulation by Streptomyces secondary metabolites may also induce cyclophilin gene expression. overall, stress related genes such as Cyclophilin 1 and Cytochrome P450 are very complex by nature because many of the enzymes have multifunctional roles in various cell processes (Lehr et al. 2009).

5.4 Microscopy observations

The microscopic observations of *H. annosum* grown in dual culture against *Streptomyces* sp. A11 indicates morphological changes, suggesting that fungal hyphae were under stress. This is in agreement with results from other experiments in this study showing that *H. annosum* growth was inhibited in dual culture against *Streptomyces* sp. A11.

Stress on fungal hyphae appears before visible inhibition of growth suggesting that secondary metabolites produced by *Streptomyces* sp. A11 were affecting *H. annosum* prior to visible growth inhibition and formation of thicker hyphae around inhibition zone (from macroscopic observations). Similar signs of fungal suppression in dual culture was observed under the microscope by Yang et al. (2015). In the mentioned study, take-all pathogen, *Gaeumannomyces graminis* var. tritici hyphae appeared to be branching, distorted and devoid of cytoplasm when paired against isolates of *Bacillus subtilis* strains.

The aim of the microscopic observation was to observe morphological changes of both *H. annosum* and *Streptomyces* sp. A11 cells in dual culture. Although the observation experiment was a valuable addition to other results in this study, it is difficult to make precise conclusions based on the microscopy observations. This is partly because microscopic observations on this level do not produce quantitative scientific data that can be challenged statistically against a hypothesis. Additionally, since microscopic observation and image capturing is done manually, it is difficult to not have biases on selecting what parts of the culture to observe (under the microscope) and capture as images.

5.5 Technical Considerations in Experimental Design

5.5.1 Streptomyces strain selection

As mentioned in section 3.1, the *Streptomyces* strains used in this study were isolated from the mycorrhizal fungi of Japanese red pine in Japan rather than Scots pine which is the dominant pine species in Finland forests. However, there are potentials in

studying these interactions despite *Streptomyces* strains not being isolated from native pines. As stated earlier, *Streptomyces* species have previously been isolated from mycorrhizal fungi of *P. sylvestris* in Europe (Maier et al. 2004) hence, there are other related strains of *Streptomyces* bacteria occupying the same or similar ecological niches with Scots pine. Marupakula et al. (2016) examined individual root tips of *P. sylvestris* at different time points for ECM and bacterial communities using sanger and pyrosequencing. Results showed that different ectomycorrhizospheres have similar bacterial communities at the phylum and genus levels. However, communities were distinctly different at the Operational taxonomic unit (OTU) level. Thus, despite the large geographical differences, the *Streptomyces* species isolated from pine mycorrhizae of diverse geographical regions may possess many similarities. Moreover, as discussed in chapter 1.4, several studies demonstrate how specific and widely different *Streptomyces* strains can impact plants or fungi species in their environment. A single strain of *Streptomyces* can have highly specific responses to different species or different strains of a single species (Lehr et al. 2007). Therefore, it is valuable to study diverse range of *Streptomyces* strains in order to find strains with the highest potential as bio-control agents.

5.5.2 Gene expression and its interpretations

Studying gene expression in microbes reveals various aspects of an organism and the conditions it is facing. Understanding how an organism responds to biotic or environmental changes on gene expression level is a powerful tool. nevertheless, results from quantitative PCR alone is inadequate for confident conclusions. This due to our limited knowledge of genome of most species. Furthermore, many genes belong to a big gene clusters that can have diverse range of functions within the organism. Thus, having the results of how a treatment affects gene expression in an organism may not be conclusive on its own. Rather, genomic and non-genomic tests should be used concurrently in order to provide a broader understanding of the studied organism and the impacts of the tested treatments on the test subject. In this project, the aim of gene expression tests on *H. annosum* was to have a broad understanding of impact of growth inhibition on group of genes belonging to different cell functions.

On a personal point, it was an essential learning experience to set up a gene expression tests from beginning end and to gain experience in all different aspects of this emerging technology. From planning, selecting and designing primers to performing all the laboratory tasks and interpret and calculating the data produced from the experiment.

5.5.3 Limitations and future studies

In the soil microcosm experiment, the fresh weight of the seedlings was measured rather than the more accurate measurement of the dried weight. This was done because the harvested seedlings were used for RNA extraction after measurements of their weight and lateral roots. RNA is very unstable and degenerates faster than DNA, therefore, it would have not been possible to use dried seedlings for RNA extraction. A better solution would be to set up parallel different sets of microcosms for different measurement to ensure the measurement dried weight of pine seedlings.

For fresh weight and lateral root formation, data of each week were analysed independently. This is because similar treatments from different time intervals did not follow any growth trajectory. This may be as result of diverse genotypes and phenotypes of each seedling or abiotic factors that can affect the growth of seedlings at early stages. Further, limited sample size may have contributed to it. As a result, no comparison was made between data sets of treatments harvested at different time points. Therefore, the results of treatments from each week were analysed independently for each week. The microcosm plates for each time interval was setup simultaneously. Thus, they have similar soil and abiotic factors.

Figures 5 and 7 were produced to visualise the overall average of results for seedling fresh weight and lateral root formation over the 4 weeks that samples were harvested. The aim of the graphs was to simplify understanding the results of all 4 weeks. There would be no point to conduct further statistical analysis on the overall data since data was not uniformly collected (different time points).

Having this study in the context of master's thesis meant that there were limitations in time and resources for setting up experiments. In a broader study, the mycorrhizal fungi (*T. matsutake*) would be included in the experiment and all the possible

interactions of other species, with and without the mycorrhizae would be studied. Additionally, in an improved study, a number of *Streptomyces* and *H. annosum* strains would be tested rather than a single strain. This would provide a broader understanding of the complex interactions between these microbes.

Future studies should also aim to move from *in vitro* experiments, to tests that closely resemble natural rhizosphere of forests where large communities of microbes remain in a balanced competition for resources and influence each other through production of metabolites. *In vitro* antagonism may be insufficient for the selection of biocontrol strains since *streptomycees* species can play many roles, such as inducers and suppressors of plant defence responses, as well as plant growth promoters (Schrey and Tarkka 2008). Despite the limitations, the results from this study still holds value as it can be used as a preliminary study and it can be built upon to assemble a detailed investigation in the future.

6. Conclusion

This study aimed to have a better understanding of the relationship between pathogenic fungi *H. annosum* and the mycorrhiza-associated bacteria, *Streptomyces* sp. A11 in different settings. *Streptomyces* sp. A11 proved to suppress growth of *H. annosum* in dual culture setup. Potentially by producing antifungal secondary metabolites that cause disruption in glucose metabolism and cell wall integrity of *H. annosum*. *Streptomyces* sp. A11 did not improve pine seedling's growth and root development in a microcosm set up. Moreover, pine seedlings inoculated with both *Streptomyces* sp. A11 and *H. annosum* had more severe infection compared to pines infected with *H. annosum* alone. This implies that Streptomyces sp. A11 has a role in regulating pine's defence response during interaction with *H. annosum*.

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